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INFORME TRIMESTRAL DEL PROYECTO DE CONSERVACIÓN EX SITU DE ESPECIES DE INTÉRES DE ANFIBIOS

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INFORME TRIMESTRAL DEL PROYECTO

"LA CONSERVACIÓN EX SITU DE RANAS DEL ÁREA DE DONOSO, RENOVACIÓN POR TRES AÑOS"

Período: Mayo-Julio 2020

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Introducción

El 1 de septiembre de 2019, Minera Panamá S.A. (MPSA) y el Instituto Smithsonian de Investigaciones Tropicales (STRI) firmaron un acuerdo con el fin de extender el proyecto de conservación ex situ de anuros del área de Donoso por tres años. Con este acuerdo se continuarán los esfuerzos de conservación e investigación que contribuirán al manejo y preservación de los anfibios de Donoso, enfocándose en las especies de interés (EdI). Este proyecto es coordinado por el Proyecto de Rescate y Conservación de Anfibios de Panamá (PARC), que lleva a cabo las actividades de conservación ex situ dentro de instalaciones del STRI, ubicadas en Gamboa, provincia de Colón. En este informe se presentan los avances, de acuerdo a los objetivos acordados de este proyecto, realizados durante el período Mayo-Julio 2020.

Durante este trimestre, a pesar de las limitaciones resultado de la pandemia causada por el COVID-19 y de la cuarentena que implica la suspensión de operaciones de un gran número de actividades consideradas no esenciales, nosotros hemos podido manejar la situación y continuamos brindándole el todo cuidado requerido a las poblaciones de anfibios que mantenemos en el programa de conservación ex situ en Gamboa; al mismo tiempo, evitando o minimizando el riesgo la salud de nuestros colaboradores. Estas actividades se realizaron siguiendo las regulaciones y la autorización de las entidades de gobierno correspondientes. No obstante, varias actividades adicionales fueron interrumpidas; las cuales, no están directamente vinculadas al cuidado y reproducción de los animales, como aquellas que están relacionadas con trabajo de campo. Estas actividades se tendrán que planificar y reprogramar en el futuro, cuando se retorne a la normalidad laboral en el país.

Objetivo 1. – Mantener la instalación para la cría y reproducción de poblaciones en cautividad de las especies de anfibios de interés de Donoso.

Debido a las restricciones por la pandemia del COVID-19, no ha sido posible seguir invirtiendo en la capacitación de personal; por lo que, no hemos tenido voluntarios, estudiantes, pasantes ni colaboradores de investigación en nuestras instalaciones durante estos meses. Además, en ocasiones, ha sido necesario trabajar con un número muy reducido de personal en el cuidado de los anfibios; ya que, cinco de nuestros colaboradores estuvieron aislados preventivamente por un par de semanas. No obstante, nos esforzamos para completar las labores básicas que se requirieron para mantener a los anfibios adecuadamente.

Realizamos trabajos de mantenimiento y reparación de equipo en la instalación de Gamboa, principalmente relacionados a la inspección, limpieza y reemplazo de aires acondicionados, cambio de filtros de agua y el mantenimiento del generador eléctrico y de la planta de tratamiento de aguas residuales. Continuamos con los trabajos de equipamiento y habilitación del último contenedor para maximizar el espacio disponible para mantener anfibios en Gamboa, particularmente en la adecuación de tanques de reproducción. Adicionalmente, a causa de un accidente en el Canal de Panamá por la colisión de un barco con el puente de Gamboa, la tubería de abastecimiento de agua potable fue dañada; lo cual, produjo una interrupción del suministro normal de agua por aproximadamente una semana en Gamboa. Fue necesario responder rápidamente a esta situación, para evitar la falta de agua en nuestras instalaciones; para lo cual, adquirimos e instalamos un tanque reservorio de agua, bomba de agua y tuberías. Este tanque de agua era llenado diariamente por un camión cisterna, permitiendo distribuir agua a los contenedores con anfibios hasta que se reestableció el servicio normal de agua potable en Gamboa. Los trabajos de construcción de la pequeña edificación (150 m^2) destinada a la producción de invertebrados, por la empresa Kunkel Constructions Inc., continúan suspendidos desde mediados de marzo, a causa de la cuarentena por COVID-19. Aún estamos a la espera de noticias adicionales sobre el refrendo del convenio, derivado de la selección de nuestro proyecto por el Ministerio de Ambiente (MiAmbiente) en diciembre 2018, que financiará parcialmente la construcción del edificio para la producción de invertebrados.

La producción de invertebrados continuó sin contratiempos e ininterrumpidamente en los salones temporales, ubicados en el área conocida como Santa Cruz. Continuamos produciendo grillos, dos especies de moscas de las frutas, colémbolos, larvas de polilla, lombrices de tierra, cucarachas, larvas de escarabajos (“mealworms”), cochinillas y tenebrios para alimentar a las poblaciones de anfibios en cautiverio. Durante este trimestre, la producción de estos invertebrados sobrepasó el nivel requerido como alimento de las ranas que tenemos en Gamboa, lo cual es adecuado en caso de que ocurra una caída repentina en la producción de alimento para los anfibios.

Los números de individuos de las poblaciones en cautiverio de las especies de anfibios del área de Donoso, que manteníamos al 31 de julio de 2020, en Gamboa eran:

Especie	Machos	Hembras	Juveniles	Total	Reproducción en cautiverio	Renacuajos
Andinobates geminisae	31	56	90	177	+	~25
Atelopus varius	282	232	100	614	+	~1000
Craugastor evanesco	21	19	25	65	-	-
Gastrotheca cornuta	9	2	0	11	-	-
Oophaga vicentei	21	26	3	50	+	~2
Total	**364**	**335**	**218**	**917**		**~1027**

Nota: *Craugastor evanesco* y *Gastrotheca cornuta* no presentan etapa de larvaria o renacuajo libre.

Al 31 de julio de 2020, teníamos 917 ranas de cinco especies de anuros del área de Donoso. Entre las especies, que se mantienen en cautiverio, están cinco Especies de Interés (EdI) del Proyecto Mina de Cobre Panamá: *Andinobates geminisae*, *Atelopus varius*, *Craugastor evanesco, Gastrotheca cornuta* y *Oophaga vicentei*. Esta última ha sido recientemente

considerada como una especie en peligro por la Lista Roja de Especies Amenazadas de la UICN, principalmente debido a que se trata de una especie endémica con una distribución restringida.

Para las especies *Andinobates geminisae*, *Craugastor evanesco y Oophaga vicentei* originalmente se colectaron suficientes individuos fundadores para iniciar la población ex situ. Sin embargo, es recomendable obtener más individuos de *Atelopus varius* (6-10 hembras de la población en Nuevo Petaquilla) y *Gastrotheca cornuta* (13 machos y 19 hembras) para obtener una representatividad adecuada de su diversidad genética. La especie *G. cornuta* sido particularmente difícil de encontrar; por lo que, nos estaremos concentrando en la búsqueda de más individuos de esta especie. Considerando las circunstancias actuales, esperamos reanudar expediciones el próximo año, con el fin de recolectar más fundadores de esta especie.

Macho de *Andinobates geminisae*, transportando un renacuajo en el dorso.

Durante este período, se mantuvieron 26 parejas de *Andinobates geminisae* en tanques y hubo varios eventos de reproducción de estas parejas. Resultado de los eventos reproductivos, al final del período, se tenían unos 25 renacuajos y 90 juveniles. Adicionalmente, mantuvimos 23 parejas de *Oophaga vicentei* durante este período, que produjeron tres juveniles saludables y hemos observado un par de renacuajos entre las plantas bromelias. Debido a que no es recomendable manipular las bromelias para evitar la interrupción del cuidado parental de los huevos y renacuajos, no podemos establecer si hay más de éstos entre las plantas. Estamos por reemplazar las bromelias naturales por artificiales, recomendadas especialmente para la reproducción de ranas dendrobátidas; las cuales, ya hemos probado en tanques con parejas de *O. vicentei* y hemos verificado que las hembras las utilizan para depositar sus renacuajos. El uso de bromelias artificiales disminuirá el tiempo de trabajo que se requiere para mantener sitios de deposición de huevos y renacuajos, evitando la búsqueda, recolección, limpieza y reemplazo constante de las plantas naturales y, así dejar, que las ranas se reproduzcan sin perturbaciones.

Renacuajo de *Oophaga vicentei* que fue depositado en una bromelia artificial y que está bajo el cuidado de la madre, la cual deposita huevos infértiles para alimentarlo.

Además, se mantuvieron 11 parejas de adultos de *Craugastor evanesco* en tanques para su reproducción; pero, aunque una pareja estuvo en abrazo nupcial en este período, éstas no se reprodujeron. A partir de la puesta de *Atelopus varius* reportada en el trimestre pasado, se obtuvieron únicamente dos post-metamorfos. Durante este período, se colocaron 10 parejas de *A. varius* en tanques de reproducción, de las cuales solamente dos parejas depositaron huevos. De la primera puesta de huevos eclosionaron unos 1000 renacuajos; mientras que, los embriones de la segunda puesta se están desarrollando normalmente. El resto de estas parejas no puso huevos o colocaron huevos infértiles, que no se desarrollaron. Además, se mantuvieron dos parejas de *Gastrotheca cornuta*; pero, todavía no se han reproducido, a pesar de que los machos emiten llamados para atraer a las hembras, que ya están grávidas. A través de estos intentos y eventos reproductivos, seguimos conformando paulatinamente poblaciones estables en cautiverio de las especies EdI de Donoso.

Comportamiento de cortejo en una pareja de *Oophaga vicentei*.

El síndrome de patas débiles (SLS) en los renacuajos y post-metamorfos de las especies, que se están reproduciendo en Gamboa, se no fue observado en este período; lo cual, constituye un logro, ya que este síndrome impide el movimiento normal para una adecuada alimentación y conlleva a la muerte de los individuos post-metamorfos. La causa de este síndrome ha estado siendo investigada por nosotros. Actualmente, sabemos no es debida a diferencias en el contenido proteínico de su dieta, lo cual determinamos experimentalmente en un estudio que publicamos el año pasado. Además, recientemente, publicamos los resultados del estudio experimental que realizamos sobre el SLS en renacuajos de *Atelopus varius*, donde se varió la proporción de calcio y fósforo presentes del agua en seis tratamientos diferentes del experimento. El artículo publicado se titula: "Spindly leg syndrome in *Atelopus varius* is linked to environmental calcium and phosphate availability", en el cual se muestra que existe una relación clara del calcio y, en menor grado, del fosfato con la aparición del SLS (Anexo 1). Sin embargo, no pudimos eliminar completamente la aparición de este síndrome, lo que sugiere que puede haber una interacción con otros factores no identificados en este experimento. Un manuscrito adicional acerca de los resultados un experimento sobre los efectos que tienen el grado de cuidado y la calidad del agua en la prevalencia del SLS en renacuajos de *Andinobates geminisae*, fue sometido por Kathleen Higgins (quien fue una de nuestras pasantes) para su publicación en una revista científica. Este manuscrito se encuentra en el proceso de evaluación por el editor y revisores de la revista.

Algunos de los 1000 renacuajos de *Atelopus varius* de la puesta reciente, alimentándose.

Además, se ha continuado con la introducción de datos de los individuos de Donoso en la base de datos "Zoological Information Management System" (ZIMS) (http://www2.isis.org), lo cual facilita el manejo del inventario de animales, el control de la conformación genética y demográfica de las colecciones de animales vivos, la identificación de animales no relacionados para realizar cruces, el mantenimiento de un registro del estado de salud y el tratamiento que se le ha dado a cada individuo, entre otros.

Objetivo 2. – Continuar el trabajo para ensayos de liberación de anfibios en el área de Donoso.

Para este trimestre, no teníamos programado realizar ensayos de liberación de anfibios, debido a que el trabajo de campo está suspendido hasta que se pueda retornar a la normalidad. No obstante, nuestro colaborador Dr. Luke Linhoff, becario postdoctoral del Instituto Smithsonian, ha continuado analizando los datos (que se tienen hasta el momento) sobre los experimentos para determinar si las secreciones de la piel de las ranas realmente pueden disminuir o eliminar la infección por el hongo patógeno *Batrachochytrium dendrobatidis.* Ya que, se conoce que las secreciones de la piel que producen algunas especies de anfibios, en conjunto denominadas "mucosoma", inhiben el crecimiento del *B. dendrobatidis* en ensayos *in vitro*. Esto se ha observado incluso para el mucosoma de algunas especies que mantenemos en el proyecto PARC. Sin embargo, no sabemos si existe variación en las propiedades inhibitorias del mucosoma entre individuos de la misma especie o si las propiedades inhibitorias se heredan. Tampoco, se sabe con certeza si el mucosoma puede ayudar a combatir el *B. dendrobatidis* en los individuos infectados. Es por esto, que completamos un experimento *in vivo* con individuos de especies con diferente grado de susceptibilidad a este hongo patógeno, éstas son: *Agalychnis callidryas*, *Andinobates geminisae*, *Atelopus certus*, *A. glyphus*, *A. limosus* y *A. varius*. Con este primer experimento *in vivo*, se determinará si existen diferencias en la eficacia inhibitoria del mucosoma entre especies e individuos de la misma especie que están infectados por el *B. dendrobatidis*. En el caso de que el efecto inhibitorio del mucosoma contribuya a la supervivencia de las ranas infectadas, se procedería a investigar sobre la posibilidad de que estas características del mucosoma se puedan heredar. Si existe un efecto inhibitorio consistente y el mismo se hereda, podríamos seleccionar individuos cuyo mucosoma tiene propiedades inhibitorias y cruzarlos entre sí, para producir descendientes con esta característica. Estas investigaciones son particularmente relevantes para llevar a cabo liberaciones exitosas de ranas, ya que pudiésemos liberar individuos con cierta tolerancia o resistencia al hongo patógeno que se encuentra en el ambiente natural de estas especies de ranas, proporcionándoles alguna ventaja contra este hongo. Aún estamos por analizar varias muestras de piel que fueron tomadas durante el experimento *in vivo*, pero que no se han podido llevar a cabo por limitaciones para obtener los suministros de laboratorio que se requieren.

Objetivo 3. – Realizar expediciones para recolectar ciertas especies de anfibios en riesgo en el área de Donoso.

Durante este período no realizamos expediciones para recolectar individuos. Como se indicó anteriormente, *Gastrotecha cornuta* es una Especie de Interés (EdI), de la cual se requieren más individuos fundadores para establecer adecuadamente una población ex situ en Gamboa. Esperamos realizar una expedición al sitio 4CS6, luego que se reestablezca la normalidad de las operaciones en el país.

Objetivo 4. - Estudio de los hongos patógenos de anfibios en la región de Donoso.

Debido a que no realizamos expediciones a Donoso en este trimestre, no obtuvimos muestras de piel para el análisis del hongo patógeno de anfibios, *Batrachochytrium dendrobatidis*.

Anexo 1. Artículo publicado: “Lassiter, E., O. Garcés, K. Higgins, E. Baitchman, M. Evans, J. Guerrel, E. Klaphake, D. Snellgrove, R. Ibáñez y B. Gratwicke. 2020. Spindly leg syndrome in *Atelopus varius* is linked to environmental calcium and phosphate availability. PLoS ONE 15: e0235285.” https://doi.org/10.1371/journal.pone.0235285

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RESEARCH ARTICLE

Spindly leg syndrome in *Atelopus varius* is linked to environmental calcium and phosphate availability

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Abstract

Spindly leg syndrome (SLS) is a relatively common musculoskeletal abnormality associated with captive-rearing of amphibians with aquatic larvae. We conducted an experiment to investigate the role of environmental calcium and phosphate in causing SLS in tadpoles. Our 600-tadpole experiment used a fully-factorial design, rearing *Atelopus varius* tadpoles in water with either high (80mg/l $CaCO_3$), medium (50mg/l $CaCO_3$), or low calcium hardness (20mg/l $CaCO_3$), each was combined with high (1.74 mg/l PO_4) or low (0.36 mg/l PO_4) phosphate levels. We found that calcium supplementation significantly improved tadpole survival from 19% to 49% and that low calcium treatments had 60% SLS that was reduced to about 15% at the medium and high calcium treatments. Phosphate supplementation significantly reduced SLS prevalence in low calcium treatments. This experimental research clearly links SLS to the calcium: phosphate homeostatic system, but we were unable to completely eliminate the issue, suggesting an interactive role of other unidentified factors.

Introduction

Spindly leg syndrome (SLS) is a musculoskeletal abnormality commonly associated with captive-rearing of amphibian aquatic larvae, resulting in underdeveloped limbs that cannot support the body of newly metamorphosed animals [1–5]. Animals have malformed joints and reduced numbers of shortened, thinner muscle fibers in the limbs, some of which are detached [6]. The issue has been widely discussed in the amphibian husbandry community as a limiting factor for captive-breeding efforts of frogs with aquatic larvae. While there are many speculated causes of SLS, there have been few adequately replicated studies on the topic. Nutritional

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Competing interests: D. Snellgrove is employed by by Waltham Petcare Science Institute, part of Mars Petcare UK. The Waltham Petcare Science Institute provided support in the form of salary for author DS, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific role of this author is articulated in the 'author contributions' section.

deficiencies in the diet have been most widely postulated as the causative factor in SLS, but one recent study found that SLS was prevalent, even when feeding presumed nutritionally-complete diets [4].

Four independent studies suggested that soft water was related to skeletal deformities in tadpoles and metamorphs [4,6–8]. Calcium is an important mineral for tadpoles because as they metamorphose, their cartilage skeletons begin to ossify, creating a high demand for calcium [9]. The calcium is stored in endolymphatic sacs and used in metamorphosis, and the Ca plasma concentrations in larval amphibians are substantially lower than adults [9–11]. Tadpoles reared in deionized water experience poor neurological development leading to limb malformation [7], but addition of calcium improves survival [6], reduces larval deformities [8], and reduces the incidence of malformations in the limbs [7]. A previous study by our own research group found that SLS incidence was aggravated by overfeeding tadpoles, and ameliorated by filtering the water through a reverse-osmosis (RO) membrane and then reconstituting it [4]. We hypothesized that soft tap water at our facility may have led to calcium being a limiting factor and that adding calcium chloride to RO water may have reduced SLS prevalence. We also hypothesized that phosphates released from uneaten food may have altered the Ca:P ratio of the water in the overfed treatments, further aggravating the problem [4,5].

Serum calcium and phosphate concentrations are homeostatically regulated through intestinal absorption, bone mineral deposition, and kidney mineral excretion (DiMeglio and Imel, 2019). When serum concentrations of Ca are low, parathyroid hormone increases intestinal absorption, bone and renal resorption (Blaine et al., 2015; DiMeglio and Imel, 2019). Inadequate Ca:P ratios in diets or factors affecting the regulation of calcium metabolism such as insufficient UVb exposure and vitamin D_3 deficiencies have been associated with symptoms of a metabolic bone disease in adult amphibians [9,12–14]. While dietary Ca:P ratios of 1:1–1.2 are recommended for feeding adult reptiles and amphibians [14], it is unclear whether high phosphate concentrations in the water affects calcium metabolism in tadpoles. Experimental evaluations of phosphate toxicity found no effect on tadpole survival, growth or development [15], but there is a perception among amphibian husbandry professionals that excessive phosphates are undesirable for tadpole development [16].

We conducted a controlled, laboratory-rearing experiment to determine the effects of calcium and phosphate concentrations on the prevalence of SLS in *Atelopus* tadpoles. We hypothesized that SLS is induced by insufficient bodily stores of calcium for metamorphosis, or an imbalance in calcium: phosphate ratios in the water.

Methods

Experimental design

We used a fully-factorial design with high, medium, and low calcium, each replicated with high or low phosphate levels for a total of six treatment groups. Each treatment was replicated in five tanks with 20 tadpoles per tank for a total of 30 tanks and 600 *Atelopus varius* tadpoles. Water for each treatment was prepared by filtering tap water (through an AquaFX 100 GPD reverse osmosis membrane) that removed 80–90% of the total dissolved solids. The RO water was reconstituted using 46.5 mg $MgSO_4$ 35.8 mg $KHCO_3$, and 29.8 mg $NaHCO_3$ per liter following Association of Zoos and Aquariums (AZA) RO reconstitution recipe [17]. Calcium treatments were prepared with 0 (low), 39.5 (medium), or 79 (high) mg $CaCl_2$ per liter of reconstituted water. The same medium calcium concentrations are recommended in the AZA recipe used by Camperio Ciani et. al. [4], and we doubled that concentration for the high treatment. The phosphate treatments were obtained by adding either 0 or 2.71 mg Na_3PO_4 per liter of water for the low and high treatments respectively. We selected a quantity of sodium

orthophosphate that would increase the phosphorous (P) concentration in the RO water by 0.5mg/l. About 87% of natural streams in the US have phosphorous (P) concentrations lower than 0.5mg/l, which is considered a high concentration in streams [18]. As reference to natural conditions, calcium hardness and phosphate levels were measured in 2 streams with healthy populations of *Atelopus varius*; which were determined to be classified as "soft" (14.2– 18mg/l hardness) and low in phosphates (0–0.07 mg/l) (S1 Data, *Atelopus* Stream Water Quality). This design received approval from the Smithsonian Tropical Research Institute's Animal Care and Use Committee 2018-0427-2021.

Husbandry

A clutch of *Atelopus varius* eggs was laid at the Panama Amphibian Rescue and Conservation Project's Gamboa facility on Jan 10, 2019 and reared in that tank containing carbon-filtered tap water for 27 days until Feb 6, 2019. We then collected 600 *Atelopus varius* tadpoles from the same clutch of eggs and divided into thirty randomly selected groups of 20 tadpoles at Gosner stage 20–25 [19] before placing them in the 20-litre aquariums 40cm long x 20 cm wide x 25 cm high without any gravel or lighting other than ambient fluorescent ceiling lights. Water was filtered using a small Hikari Aquarium Solutions Bacto-Surge Foam Filter with an airstone in each tank plus one supplemental air stone to improve circulation. Water was partially (30%) changed three times per week through siphoning. Each tank was fed 0.125g of a nutritionally-balanced food developed at the Waltham Center for Pet Nutrition that contained 1.8% calcium (Table 1). The food was weighed then mixed with water to make a paste that was spread to ~1mm thickness on an acrylic plate and dried as a film. Each day the feeding plates were replaced in each tank. Once per month, immediately prior to water changes, $CaCO_3$ and PO_4 levels were measured using HANNA HI720 and HI713 test kits. For all tanks, the mean temperature was 20.9˚C +/- 0.7 ˚C SD (stream temperatures at sites where parents were collected ranged from 22–26˚C RI, unpublished data); the mean pH 7.5 +/- 0.2 SD; the mean dissolved oxygen 89.7% +/- 3.7% SD; the mean NH_4 2.1 mg/l +/- 1.1 mg/l SD; and NH_3 was not detected (S1 Data, Water Quality).

When tadpoles developed hind limbs, floating polystyrene slices were placed in the tanks to facilitate froglet emergence. For each tank we recorded 1) prevalence of SLS by classifying each metamorph into 'spindly' or 'healthy'. All SLS frogs exhibited in SLS in the fore-limbs only (Claunch and Augustine, 2015, Fig 1); 2) mortality or survival through metamorphosis Gosner Stage 46, [19]; and 3) snout-vent-length (SVL) and weights of metamorphs. Any animals with SLS were euthanized by 20% benzocaine gel applied directly to the skin as advocated in AVMA humane euthanasia guidelines (AVMA, 2007).

Statistical analysis

A previous study demonstrated that this sample size was sufficient to highlight a 10% difference in survival between groups with a power of 80% [4]. Data were visualized by plotting mean values for each tank, which served as the experimental unit, with corresponding standard error bars. We examined data for assumptions of normality and homogeneity of variance by examining residual plots, difference showing $P<0.05$ were considered significant. Only animals reaching metamorphosis are included in the statistical analysis. The independent effects of calcium and phosphate, and their interaction were modeled using a linear model in R with the package 'car' testing the experimental treatment effects on the following response variables; 1) SLS prevalence in metamorphs; 2) tadpole survival to metamorphosis; and 3) size of metamorph measured by SVL and weight. Model = lm("responsevariable" ~ phosphate + calcium + phosphate: calcium, data = data), results were reported as a type II ANOVA [20]. We used

Table 1. Nutritional analysis of tadpole food (% dry matter basis) batch prepared by the Waltham Petcare Science Institute.

Analysis		Unit
Proximates		
Ash	11.7	%
Crude Fibre	1.8	%
Fat	7.5	%
Moisture	3.2	%
Protein	38.5	%
Vitamins		
Vitamin B_1	28.8	mg/kg
Vitamin B_2	41.1	mg/kg
Vitamin D_3	4790	IU/kg
Vitamin A	30400	IU/kg
Vitamin E	510	mg/kg
Pantothenic acid	163	mg/kg
K3	15.9	mg/kg
Niacin (B_3)	391	mg/kg
Choline	2910	mg/kg
B_6	28.9	mg/kg
B_{12}	0.084	mg/kg
Folic acid	14.4	mg/kg
Vitamin C	2220	mg/kg
Minerals		
Copper	20.3	mg/kg
Calcium	1.82	%
Magnesium	0.187	%
Manganese	73.7	mg/kg
Iron	723	mg/kg
Potassium	0.764	%
Sodium	0.772	%
Zinc	96.7	mg/kg
Phosphorus	1.12	%
Essential Fatty Acids		
Linoleic	0.578	%
Linolenic	0.087	%
Arachidonic	0.055	%
Eicosapentaenoic	0.343	%
Docosahexaenoic	0.301	%
Amino Acids		
Aspartic acid	3.15	%
Serine	1.54	%
Glutamic Acid	6.15	%
Glycine	2.4	%
Histidine	0.8	%
Arginine	2.19	%
Threonine	1.43	%
Alanine	2.05	%
Proline	2.14	%

(*Continued*)

Table 1. (Continued)

Analysis		Unit
Tyrosine	1.12	%
Valine	1.63	%
Methionine - Total Analysis	1	%
Lysine	2.49	%
Isoleucine	1.4	%
Leucine	2.53	%
Phenylalanine	1.5	%
Cysteine	0.37	%

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survdiff in the R package survival for R [21] to conduct a log-rank test [22] for differences in survivorship between the 6 treatment groups.

Results

The addition of calcium to create a mean calcium hardness of ≥50mg/l markedly improved *Atelopus varius* tadpole survival to metamorphosis ($P < 0.0001$) (Fig 2, Table 2). Overall, the four treatments with added calcium experienced 49% survivorship, but this was reduced to 19% in the treatment groups with no additional calcium which had a mean calcium hardness of about 20mg/l (Fig 2). The addition of phosphate had no significant effect on survival ($P = 0.1461$, Table 1), and there was no significant interaction between Ca and P. A survivorship analysis indicated that the largest differences from expected values were in the medium Ca, added PO4 treatments and the two low Ca treatments (Fig 3, Table 3).

Adding calcium was associated with a highly significant reduction in SLS prevalence from about 60% without supplemented calcium to 15% in high and medium calcium-supplemented treatments ($P < 0.0001$, Fig 2, Table 1). As with survival, it appears that once a threshold of 50mg/l $CaCO_3$ was reached, additional calcium did not further reduce the incidence of SLS. The addition of phosphate to the water also reduced SLS prevalence in the low-calcium treatment ($P < 0.05$, Fig 2, Table 1). Overall, SLS prevalence averaged 23% in groups with added phosphate and 39% in groups with no added phosphate.

Fig 1. ***Atelopus varius*** **metamorphs: Typical spindly leg syndrome posture (left) and normal, healthy posture (right).**

https://doi.org/10.1371/journal.pone.0235285.g001

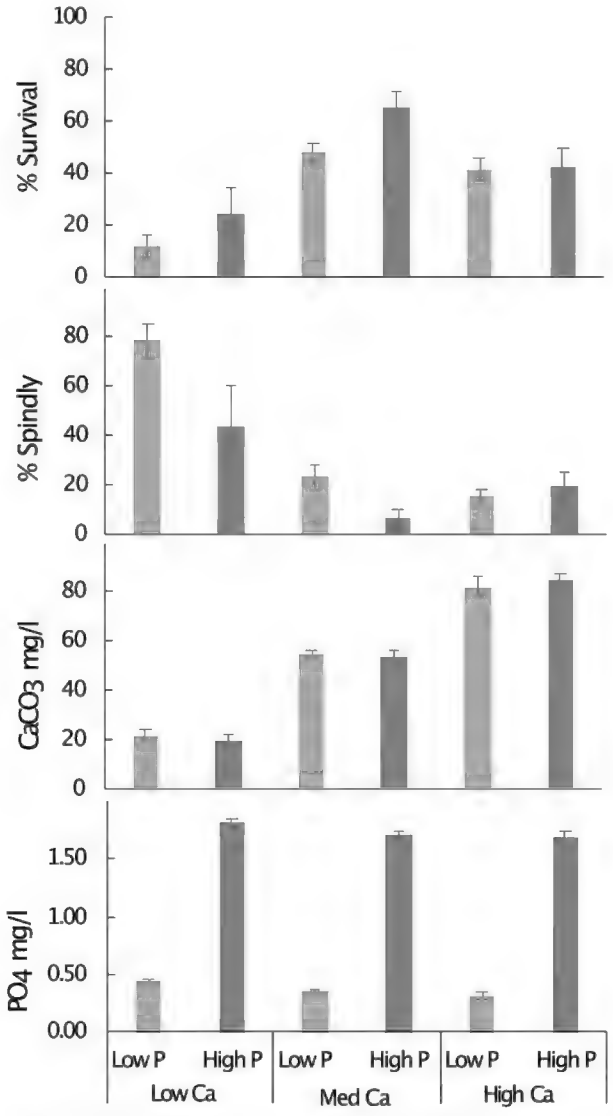

Fig 2. Effects of phosphate and calcium on survival to metamorphosis and prevalence of spindly leg syndrome among surviving *Atelopus varius* metamorphs. Shown are means +/- SE (bars). Each treatment was replicated in 5 tanks of 20 tadpoles, $CaCO_3$ and PO_4 was measured monthly in each tank for 4 months.

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Table 2. 2-way ANOVA testing for the experimental effects of added calcium and phosphate on *Atelopus varius* tadpole survival, and prevalence of spindly leg syndrome in *A. varius* metamorphs.

% Survival				
	Sum of Squares	DF	F Value	Probability
Phosphate	0.0507	1	2.263	0.1461
Calcium	0.6912	2	5.406	0.00006 ***
PhosphateCalcium	0.0326	2	0.726	0.4942
% Spindly Leg Syndrome				
	Sum of Squares	DF	F Value	Probability
Phosphate	0.1729	1	4.817	0.038 *
Calcium	1.2135	2	16.90	0.00003 ***
Phosphate*Calcium	0.1739	2	2.422	0.111

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There was no significant effect of any treatment on the size or weight of froglets at metamorphosis which took 105 days +/- 15 days (SD). *Atelopus varius* size at emergence measured 8 mm SVL +/- 0.6mm SE, and weighed 0.086 g +/- 0.06g SE and SLS metamorphs did not significantly differ from healthy metamorphs in weight, length, or number of days to metamorphosis (two sample t test, $P > 0.05$, S1 Data, Experimental Data).

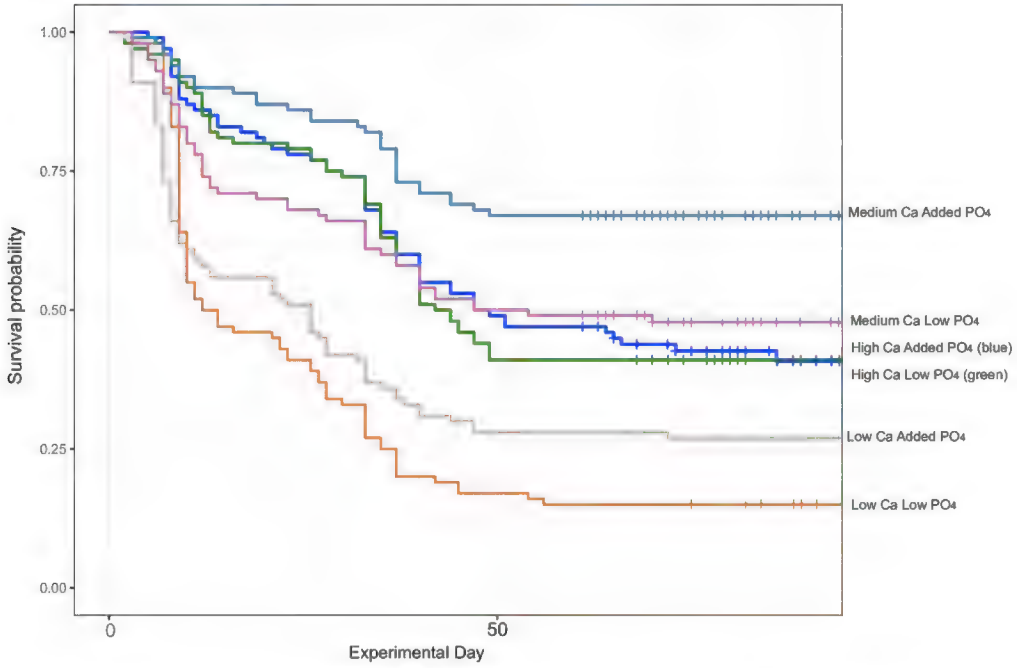

Fig 3. Survival probability curves for the 6 experimental treatment groups, cross hatches indicate censored animal(s) that metamorphosed and were censored from this survivorship analysis at that point. A log-rank test (Table 3) revealed highly significant differences between groups, Chisq = 94 on 5 degrees of freedom, p = <2e-16.

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Table 3. Log-Rank test for differences in survival probability between the 6 treatment groups. Chisq = 94 on 5 degrees of freedom, p = <2e-16.

	N	Observed	Expected	(O-E)^2/V	(O-E)^2/E
High Ca Added PO4	100	58	68.4	1.59	2.04
High Ca Low PO4	100	59	67	0.964	1.23
Low Ca Added PO4	100	75	46.5	17.417	20.79
Low Ca Low PO4	100	88	44.8	41.69	51.75
Medium Ca Added PO4	100	34	75.5	22.82	30.13
Medium Ca Low PO4	100	52	63.7	2.148	2.71

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Discussion

Even though the diet offered to tadpoles contained 1.8% calcium, we found lower tadpole survival and increased prevalence of SLS in water with calcium hardness lower than 50mg/l. Tadpoles absorb calcium from their environment primarily across the gills (70%) and skin (25%) [23], thus the supplementation of calcium to the water is likely more effective for tadpoles rather than by diet. Others have reported that limitation of calcium in the rearing water is directly correlated with the accumulation of calcium in endolymphatic sacs and skeletal mineralization [11]. Our observed threshold for calcium hardness as a limiting factor was between 20–50mg/l $CaCO_3$ for *Atelopus varius*. Water hardness of the tap water in the facility fluctuated from 5–50 mg/l (S1 Data, Source Water Quality), levels classified by the United States Geological Survey as "soft". This may explain some of the observed variation in SLS prevalence over time. Supplementation of filtered tap water with $CaCl_2$ was more cost-effective than preparing fully reconstituted RO water. It is unclear whether the observed $CaCO_3$ threshold will apply to other taxa or situations. Haakvoort et al. [6]. reported 100% cases of SLS in dart frogs at 100, 10 and 1 mg/l $CaCO_3$.

Water supplementation of phosphate was associated with a significant reduction of SLS. The experimental effect, however, was less pronounced than the supplementation of calcium (Fig 2, Table 2), and phosphate supplementation did not significantly affect survivorship. This finding provides further evidence that SLS, at least in *Atelopus varius*, is connected to an imbalance in calcium and phosphate homeostasis, but it suggests that the hypothesis that high phosphate levels and high calcium: phosphorous ratios in the water are the not the cause of SLS in this species.

In this experiment, even the unsupplemented group's water would fall within the ranges of those parameters in the two natural reference streams. Experimentally, calcium water supplementation accounted for most of the observed variation in survival and SLS prevalence, but there still was 10–20% SLS in calcium-supplemented treatments; suggesting a potential role of some other factor, such as overfeeding [4], which was untested in this experimental design. Alternatively, SLS may be a more common condition in nature than is currently appreciated, there is at least one anecdotal report of SLS in the wild *Ranitomeya ventrimaculata* from Río Napo/ Ecuador (pers. comm. H. Divossen).

All tadpoles were fed the same quantity of food, but we have not yet determined a feeding regime where food quantity limits growth. It is possible, therefore, that all tadpoles in this experiment were overfed as uneaten food remained on food plates at each change of food plates. Growth and differentiation may be partially decoupled from each other in poikilotherms through genetic modification or through rapid growth associated with high food availability [24]. For example, growth-enhanced transgenic salmon are known to be inferior swimmers with muscle hyperplasia and reduced muscle fiber size [25,26]. Faster growth rates are associated with reduced burst swimming speed in tadpoles [27], shorter legs in froglets

[28], and food-limited tadpoles have improved bone ossification in froglets [29]. One potential explanatory hypothesis tying these observations together is that rapid growth rates through calories absorbed in the intestinal tract could become decoupled from rates of normal sequestration of calcium across the gills, that may manifest as SLS, particularly in low calcium, low phosphate environments. The fact that we only observed SLS in the forelimbs, which are the last to emerge, may be indicative of depletion of reserves that were available to the tadpoles earlier in metamorphosis.

In conclusion, our management recommendations for husbandry and veterinary professionals seeking to reduce or eliminate SLS in anurans are to 1) test water hardness and consider supplementing water softer than 50 mg/l calcium hardness with $CaCl_2$ (this study) and 2) to avoid overfeeding tadpoles which was associated with increased SLS prevalence in an earlier study [4]. Future SLS studies may benefit by incorporating calorie limitation or examining other potentially synergistic factors that may decouple growth and development in calcium-limited environments to obtain further insights into the mechanisms responsible for SLS. A detailed comparison of husbandry and feeding protocols and water chemistry with other *Atelopus* rearing programs that have had no SLS may help to identify alternate factors.

Supporting information

S1 Data.
(XLSX)

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